THE UNIVERSITY OF ALBERTA

THYMINE METABOLISM IN *ESCHERICHIA COLI*

by

© GRANT WICKLAND

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

FACULTY OF SCIENCE

EDMONTON, ALBERTA

FALL 1969

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Thymine Metabolism in Escherichia coli" submitted by Grant Wickland, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The metabolic pathways leading to the production of deoxyribose donors have been investigated in several strains of Escherichia coli.

Phosphomonoesterase activities for five substrates (dCMP, dUMP, dTMP, dAMP, dGMP) have been examined in several wild type, high-thymine-requiring (thy^-), and low-thymine-requiring (tlr) strains. It was concluded that no significant differences in this activity were present which could explain the quantitative difference in thymine requirement.

Deoxycytidylate deaminase activity was not detected with the use of a very sensitive (10^{-2} mumole dUMP) assay employing radioactive substrate dCMP-2-^{14}C (2 uc/umole) and a polyethylenimine cellulose thin layer - 0.1M Formate (pH 3.4) system.

No direct transfer of thymine to a nucleotide donor to produce thymidylate was found in E. coli B cell-free-extracts, except in reaction mixtures producing large quantities of thymidine and containing a deoxynucleoside or nucleoside triphosphate. Both $B\text{-}1_{20}$ (thy^-) and $B\text{-}1\text{-}1_2$ (thy tlr) produced small quantities of dTMP in reaction mixtures containing dCMP and either ATP or dCTP.

Two tlr strains (5275_2 and $15TAU_2$) were investigated for loss of ability to catabolize deoxyribose compared to two wild-type strains (K12SH and B). Both tlr strains

possessed normal basal levels of deoxyriboaldolase but were deficient in deoxyribomutase activity. Neither of the tlr strains could produce dR-5-P and thus were not inducible for thymidine phosphorylase or deoxyriboaldolase when grown on thymidine or deoxyguanosine.

The high-thymine requirement of thy$^-$ mutants implies that the internal concentration of dR-1-P is too low to maintain normal DNA synthesis except at high external thymine concentrations.

The low thymine requirement of thy$^-$ tlr mutants appears to be due to the blockage of the catabolism of deoxyribose resulting in an increased deoxyribose pool and an enhanced ability to synthesize thymidine at lower external thymine concentrations.

ACKNOWLEDGEMENTS

I am grateful to Dr. W.E. Razzell for his guidance and support and to Miss Joanne Walker for the skillful typing of this thesis.

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ABBREVIATIONS

BAP	-bacterial alkaline phosphatase
DEAE	-diethyl amino ethyl
DNA	-deoxyribonucleic acid
DTT	-dithiothreitol
EDTA	-ethylenediamine tetraacetate
OD	-optical density
PCA	-perchloric acid
PEI	-polyethylenimine
TRIS	-tris-(hydroxymethyl) amino-methane
dR	-deoxyribose
dR-1-P	-deoxyribose-1-phosphate
dR-5-P	-deoxyribose-5-phosphate
C	-cytosine
U	-uracil
T	-thymine
AdR	-deoxyadenosine
CdR	-deoxycytidine
UdR	-deoxyuridine
GdR	-deoxyguanosine
IdR	-deoxyinosine
TdR	-thymidine
dAMP	-deoxyadenosine-5'-monophosphate
dCMP	-deoxycytidine-5'-monophosphate
dGMP	-deoxyguanidine-5'-monophosphate
dUMP	-deoxyuridine-5'-monophosphate
dTMP	-deoxythymidine-5'-monophosphate
dCDP	-deoxycytidine-5'-diphosphate

dATP	-deoxyadenosine-5'-triphosphate
dCTP	-deoxycytidine-5'-triphosphate
dGTP	-deoxyguanosine-5'-triphosphate
dTTP	-deoxythymidine-5'-triphosphate
ATP	-adenosine-5'-triphosphate
thy^-	-thymidylate synthetase negative
tlr	-thymine - low requirement
mg	-10^{-3} grams
mum	-10^{-9} moles
uc	-10^{-6} curies
ug	-10^{-6} grams
ul	-10^{-6} liters
uv	-ultraviolet

INTRODUCTION

Mutations in the locus controlling the biosynthesis of thymidylate synthetase cause thymine requiring mutants to occur (Cohen and Barner, 1954; Barner and Cohen, 1959). The mutant cells, in contrast to the wild type, are capable of using external thymine for growth (Crawford, 1958). All the necessary enzymes for the conversion of exogenous thymine to thymidylate appeared to be present in the wild type (Mantsavinos and Zamenhof, 1961).

Thymineless mutants die in the absence of thymine rather than enter a static condition of growth. This thymineless death has been attributed to unbalanced nuclear and cytoplasmic growth (Cohen and Barner, 1954; Maaloe and Hanawalt, 1961; Menningmann and Szybalski, 1962).

Thymineless mutants of Escherichia coli (Cohen and Barner, 1954), Aerobacter aerogenes (Harrison, 1965), Bacillus subtilus (Wilson et al, 1966) and Salmonella typhimurium (Okada et al, 1962) have been isolated. These mutants tend to fall into two groups depending on the level of thymine required in the growth medium. High thymine requirers need 20-30 ug of thymine per ml of culture medium (Harrison, 1965) compared to 0.5-1.0 ug/ml for the low-thymine requiring mutants. Both groups of mutants, however, have an identical efficiency for the

corresponding nucleoside, thymidine, requiring only 1-2 ug per ml. The thymine uptake for both high- and low-thymine requirers is the same per bacterium. Also the high-thymine requirers are about 1,000 times more sensitive to cytosine or uracil inhibition than the low thymine requirers. Super-optimal concentrations of thymine will also inhibit growth (Harrison, 1965).

It is unlikely that the high thymine requirement is a result of a permeability restriction, since the growth of the mutants can be initiated with low levels of thymine but can only be maintained by high levels of thymine (Harrison, 1965). The suggestion of a permeability restriction in high thymine requirers has also been refuted by Kammen (1967) who showed that while EDTA did relax permeability restrictions of Escherichia coli, thus allowing Actinomycin D to enter, this relaxation had no effect on the metabolism of thymine.

The examination of a tlr strain of Escherichia coli (70V3-462) implicated deoxyribose into the metabolism of thymine. Growth of the mutant on a medium lacking thymine resulted in a 10 fold increase in the specific activity of thymidine phosphorylase and the excretion of deoxyribose into the medium (Breitman and Bradford, 1964).

Deoxyribose donors promote the utilization of low concentrations of exogenous thymine by strains ordinarily dependent on high concentrations(Kammen, 1967), and an analysis of such a donor-supplemented medium after growth

showed an accumulation of the free base or its deaminated derivative associated with the deoxyribose donor employed (IdR, GdR, AdR). The induction of thymidine phosphorylase did not occur with the addition of the deoxyribose donor and the addition of chloramphenicol to prevent protein synthesis had no effect on the lowering of the thymine requirement (Kammen, 1967). Thus it appeared that the high thymine requirement was the result of an endogenous limitation on the availability of deoxyribose rather than the result of restrictions on the permeability of thymine.

It has been shown that the high thymine requirers lack the enzyme thymidylate synthetase (Barner and Cohen, 1959; Mantsavinos and Zamenhof, 1961), and that the low thymine requirement results from an additional mutation distant from the thy (thymidylate synthetase) locus in E. coli K12 (Alikhanian et al, 1966). The mutations resulting in the low-thymine-requiring strains map at approximately zero minutes (next to the threonine locus). The single thymidylate synthetase negative (thy) mutants all map next to Arg_2 distal to the thy-R or thymine-low-requirement (tlr) locus. Alikhanian et al, (1966) also reported that both high- and low-requiring mutants which they isolated could grow on 0.5-1.0 ug/ml of thymidine. This low-thymidine requirement indicated that the quantitative requirement for thymine was involved in reactions prior to those using thymidine.

Okada (1966) noted in recombination experiments carried out to determine the location of tlr (called thy-R by Okada) that recombinants receiving the thy-R gene did not express this gene until some time after incubation in fresh medium. This observation could reflect the gradual loss of some activity present in the cell prior to the recombination event.

Breitman and Bradford (1967) indicated that a tlr strain of Escherichia coli 15 (70V3) lacked deoxyribose 5-phosphate aldolase, an enzyme which converts dR-5-P to acetaldehyde and glyceraldehyde-3-phosphate (Pricer and Horecker, 1960). The loss of this enzyme could result in the accumulation of dR-1-P which Breitman and Bradford (1967) suggested would inhibit thymidine phosphorolysis. Growth of the wild type and thy_{20}^- mutants on thymidine resulted in the induction of thymidine phosphorylase and deoxyriboaldolase whereas in the double mutant (thy^- tlr^-) the aldolase was not induced and the thymidine phosphorylase was. Breitman and Bradford (1967) suggested that since the reactions catalysed by deoxyribomutase and thymidine phosphorylase are reversible and since the block at deoxyriboaldolase results in an accumulation of dR-5-P, this accumulation would lead to an increase in the intracellular level of dR-1-P and a decrease in thymidine catabolism.

$$\text{TdR} \underset{+T}{\overset{-T}{\rightleftharpoons}} \text{dR-1-P} \rightleftharpoons \text{dR-5-P} \rightleftharpoons \text{acetaldehyde} + \text{glyceraldehyde 3-P}$$

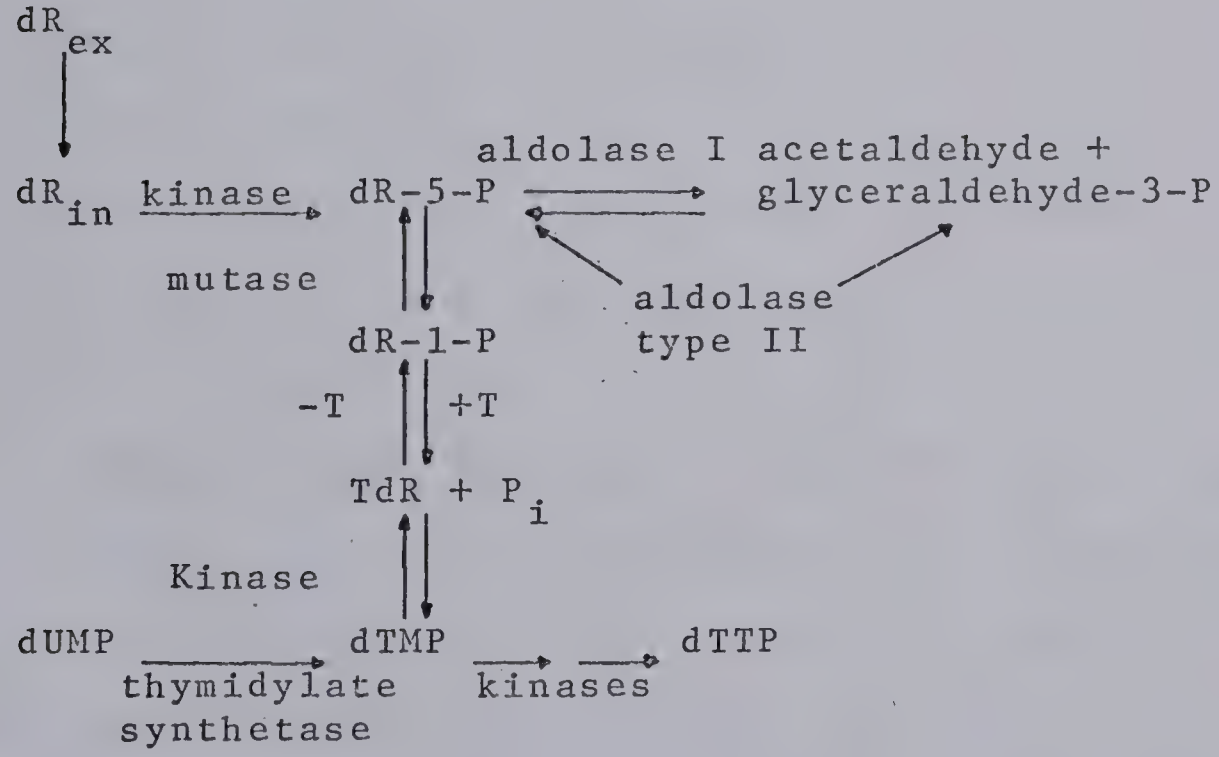
thymidine phosphorylase; dRmutase; dRaldolase

The increased levels of dR-1-P would also support increased thymidine production at lower external thymine levels.

This suggestion of an increased deoxyribose pool being responsible for the low thymine requirement was supported by investigations using Salmonella typhimurium (Hoffee, 1968). This worker claimed that Salmonella typhimurium possessed two types of deoxyriboaldolase and isolated 4 types of low thymine requiring mutants, three of which were missing one or both types of aldolase. She claimed that the type I aldolase was induced by deoxyribose and coordinately regulated with deoxyribose kinase. The type II aldolase was induced by dR-5-P and coordinately regulated with thymidine phosphorylase.

Four mutants of Salmonella typhimurium were isolated which were low-thymine requirers (2 ug/ml). Of the mutants, one (thy_2^-1) could ferment deoxyribose and contained normal induced levels of deoxyribokinase, both dR-5-P aldolases, and thymidine phosphorylase. This mutant was a low thymine requirer and yet possessed both aldolases and full aldolase activity. The thy_2^{-6} mutant could not ferment deoxyribose and was partially constitutive for thymidine phosphorylase, indicating that it was lacking both type I and type II aldolases. The

thy_2^{-21} mutant lacked the type I dR-5-P aldolase and was inducible for deoxyribose kinase but was constitutive for thymidine phosphorylase and dR-5-P aldolase type II. The fourth mutant (thy_2^{-42}) lacked the type II aldolase and was inducible for thymidine kinase and the type I aldolase but constitutive for thymidine phosphorylase. Hoffee (1968) suggested that growth on deoxyribose induced the permease, the kinase and aldolase I and that the action of these enzymes resulted in an increase in the levels of dR-5-P which in turn induced the second operon for thymidine phosphorylase and dR-5-P aldolase type II. The relationships of these enzymes are shown below.

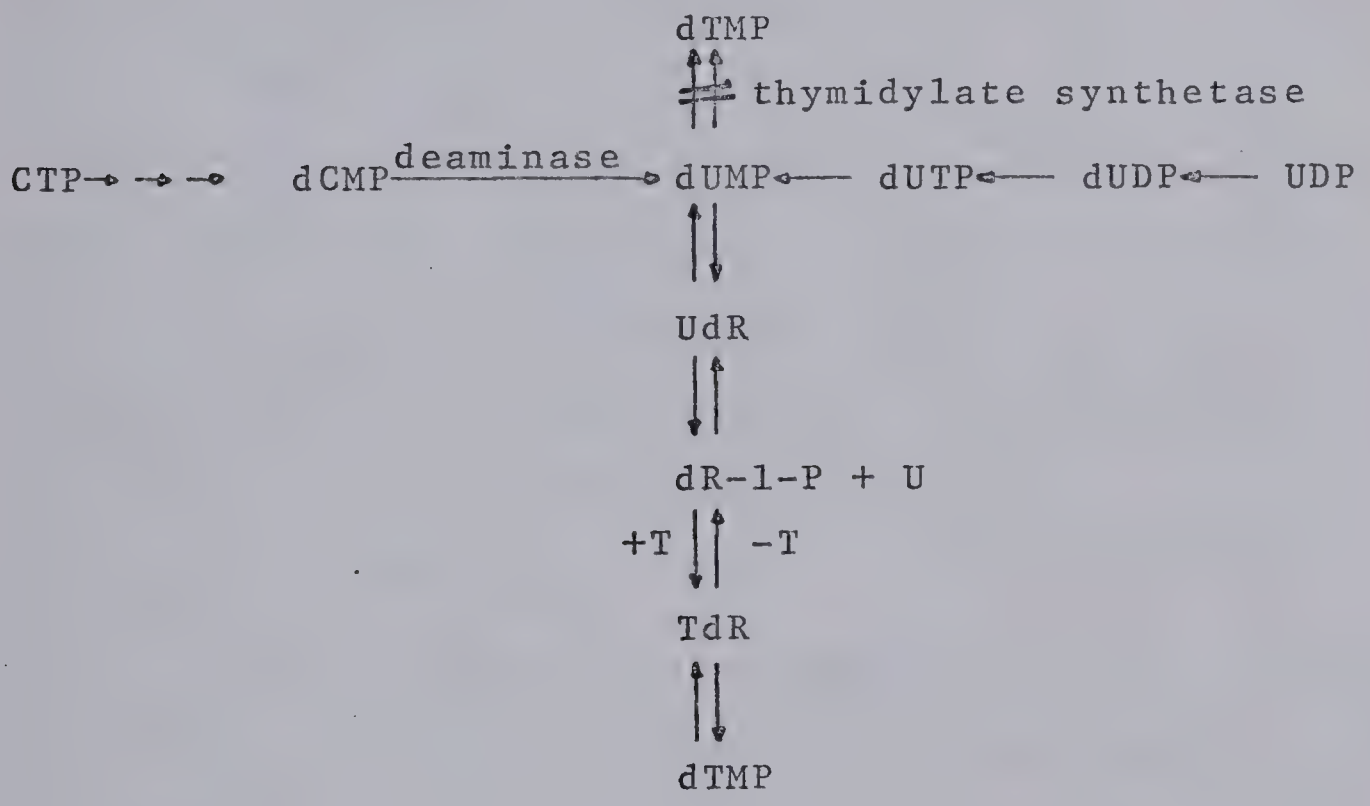


Thus if the tlr mutants lacked one or both aldolases, the catabolism of dR-5-P would slow down and dR-1-P would accumulate as the dR-5-P levels increased.

However some tlr mutants have been isolated which possess complete basal aldolase activity and cannot be induced for either thymidine phosphorylase or deoxyribo-aldolase; these include E. coli $15T^-$ and strain I (Breitman and Bradford, 1968) and thy_2^{-1} of Salmonella typhimurium (Hoffee, 1968). Breitman and Bradford (1968) attributed this phenomenon to the inability to catabolize deoxyribose-1-P due to a lack of deoxyribomutase activity. This mechanism of lowered thymine dependence is supported by a mutant, E. coli K-12-5275, used in the work for this thesis. This mutant cannot be induced by thymidine or deoxyguanosine for thymidine phosphorylase activity although it possesses normal basal levels of thymidine phosphorylase (Razzell and Casshyap, 1964) and deoxy-riboaldolase. Thus it appears that the low thymine requirement is a result of the inability to catabolize dR-1-P or dR-5-P rather than the result of an increase in the levels of deoxyribose donors by some other mechanism.

When the work compiled in this thesis was begun it was thought that a possible explanation for the ability of thymine requirers to utilize thymine was that the level of deoxyribose donors was increased over the levels which were normally found in wild type cells. In addition, evidence was available which indicated that there existed a phosphomonoesterase with greater intracellular activity for dUMP and dTMP than for any of the other deoxyribo-

nucleotides. Further evidence from previous work on the problem of thymine metabolism indicated the possibility of deoxycytidylate deaminase activity, an enzyme previously thought to be non-existent in Escherichia coli (Keck, Mahler and Fraser, 1959; Flaks and Cohen, 1959; Fleming and Bessman, 1965). Thus it seemed feasible that the low thymine requirement was brought about by an increase in the supply of deoxyribose donors by the pathways shown below.



Thus a systematic study of reactions capable of increasing the level of deoxyribose donors in thymineless mutants was carried out.

MATERIALS AND METHODS

1. Materials

All chemicals were obtained from commercial sources and were of a high standard of purity. ^{14}C-thymine was obtained from New England Nuclear Corp., Boston, Mass., and the Radiochemical Centre, Amersham. dCMP-2-^{14}C in the diammonium salt form was obtained from the New England Nuclear Corp.. Bacterial Alkaline Phosphatase was a product of Worthington Biochemicals Corp..

2. Bacterial Strains

Escherichia coli strain B was obtained from Dr. S. Luria and mutants B-1$_{20}$ and B-1-1$_2$ were isolated by R. Swanson using the aminopterin method (Stacey and Simpson, 1965). Strain 15TAU was obtained from Dr. P. Hanawalt, strain K12SH from Dr. W. Fangman and strain 5275 from Dr. J. Lederberg.

Stock cultures were maintained at room temperature in wax-sealed tubes of 1/2 strength Difco nutrient agar.

3. Media

Minimal Media

The minimal media employed contained per liter:

KH_2PO_4	2.0 g
K_2HPO_4	7.0 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4$	0.1 g
Glucose	4.0 g

The glucose and $MgSO_4$ were autoclaved together separate from the other salts to prevent carmelization.

The supplements employed were:

Thymine	20 mg	Thymine and other supplements
L-argenine	60 mg	were autoclaved separately
Uracil	10 mg	and added to the medium after
Thymidine	20 mg	sterilization.

4. Growth of Organisms

Inocula from stock cultures were plated on Nutrient Agar plates and then on either minimal plates or supplemented plates depending on the requirements of the strain. Single colonies were picked and used to inoculate suitable broth tubes which were then incubated overnight on a tube roller at 37°C. Growth was followed turbidimetrically at 650mu with a Beckman DB-G spectrophotometer. Cells were harvested in the late logarithmic phase of growth.

5. Preparation of Cell Extracts

Cells were harvested by centrifugation at 27,000 x g for 10 minutes, washed once with 0.05M Tris Acetate buffer pH 7.5 and resuspended in this buffer to yield a weight to volume ratio of 1:4. The cell suspension was kept on ice and sonicated for 30-60 seconds (2 ml quantities) using a Bronwill Biosonik (Bronwill Scientific, Rochester, New York) at full tuned power. The temperature of the cells did not rise above 10°C during this process. Unbroken cells and debris were removed by centrifugation at 20,000 x g for 10 minutes at 4°C.

The protein content of the cell free extract was determined by the method of Lowry et al (1951) using

Bovine Serum Albumin as a standard protein solution.

6. Dialysis of Cell Free Extracts

The cell free extracts were dialyzed overnight against 50-100 volumes of 0.01M Tris Acetate pH 7.5 containing 0.002M dithiothreitol.

7. Isolation of Mutants (by R. Swanson, 1968)

Strains B-1_{20} and B-1-1_2 were isolated by the aminopterin method of Stacey and Simpson (1965). Strain B-1_{20} can grow in the absence of thymine at 25°C but not at 37°C, at which temperature 20 ug/ml thymine was required for growth. Strain B-1-1_2 required 2 ug/ml thymine for growth and was isolated from strain B-1_{20} by a gradient plate technique. (Washed cells were plated on a minimal plate with about 1 mg of dry thymine in the center. After two days of incubation at 37°C, colonies on the periphery of the confluent growth were picked and tested for their ability to grow on 2 ug/ml thymine.)

8. Chromatography

(a) Paper Chromatography

Whatman No. 40 or 3MM paper was used with solvent A, isobutyric acid-ammonium hydroxide-water-0.1M EDTA (100:4.2:55.8:1.6) at room temperature employing the descending technique. Ultraviolet absorbing material was observed with short wave uv light using a Chromato-Vue (Ultraviolet Products, Inc., San Gabriel, California). The radioactivity of uv detected spots was scanned with an Actigraph III (Nuclear Chicago).

Elution of spots from paper chromatograms was accomplished by allowing distilled water to rise up the strip containing the spot. The saturated paper strip was then wrapped in a foil support and washed into a centrifuge tube in the same direction as the rise of the solvent front. The foil was folded over the lip of the tube and any remaining eluent was collected by spinning at low speed.

(b) Thin Layer Chromatography

Glass chambers were used rather than Chamber plate sets because the solvent systems employed were volatile. The standard ascending technique was used.

Polyethylenimine cellulose (Baker-flex, J. T. Baker Chemical Co., Phillipsburg, N.J.) was employed with either 0.1M Formate pH 3.4 or 2.0M Formate, pH 3.4. The polyethylenimine sheet was pre-run in distilled water to remove excess polyethylenimine which otherwise tended to obscure compounds with Rf values greater than 0.75.

Eastman Silica Gel with fluorescent indicator was used with two solvent systems:

(B) n-butanol - 20% NH_4OH, 83:17

(C) ethyl acetate - formic acid - H_2O, 45:5:50, organic phase (Fink, 1956).

The Silica Gel sheets contained a non-soluble fluorescent dye for the detection of ultraviolet-absorbing spots.

9. Liquid Scintillation Counting

Samples of radioactive material were suspended in 2.5 ml Bray's solution (Bray, 1960) and counted on a liquid scintillation counter (Mark I, Nuclear Chicago) using Channel Ratio Quench correction to determine counting efficiency.

10. Purification of dCMP and dUMP

A 1 x 3 cm column of Rexyn Ag-1 (Dowex-1 x -8) was prepared, washed with 0.5M NH_4HCO_3 and then washed with distilled water. When the pH was approximately 5.0, the column was loaded with 50 umoles of impure dCMP (or impure dUMP) and washed with water to remove contaminating nucleosides and bases. A gradient of 0.0 to 0.5M NH_4HCO_3 was then run through the column and the absorbance of the fractions read at the appropriate wavelengths (dUMP, 260 mu; dCMP, 280 mu). The fractions containing the pure nucleotide were pooled and concentrated on the rotary evaporator and then re-evaporated from distilled water to remove traces of NH_4HCO_3. The product was then dried in the desiccator and redissolved in distilled water to yield a solution of 20 umoles/ml. The yield was 62% for dCMP and 78% for dUMP. The purity of the products was checked on Silica Gel using solvent C and both products were found to be pure with no visible contaminants (less than 0.5%).

11. Deoxycytidylate Deaminase Assays

(a) The basis of the original dCMP deaminase assay used was the absorption of the substrate, dCMP, on Dowex-50

between pH 2-3 and the passage of the nonadsorbable product, presumably dUMP (Fleming and Bessman; 1965). The incubation mixture contained the following in 0.2 ml: Tris Acetate, pH 7.6, 200 mM; $MgCl_2$, 5 mM; dCTP, 2.5 mM; Dithiothreitol, 2.5 mM; dCMP-2-^{14}C, 2 mM; and about 1 mg protein.

The assay mixture was preincubated for 5 minutes at 37°C and the dialyzed cell-free extract was added at zero time. The assay mixture was sampled at 1, 5, and 15 minutes and the 50 ul samples were added individually to 0.45 ml of 0.04N HCl to yield a pH of about 2.0. This solution was then loaded onto a Dowex-50 x 2 column (H^+ form) and washed with several ml of 0.01N HCl. 0.5 ml fractions were collected and 100 ul were sampled from each fraction to be counted in 2.5 ml Bray's solution in the Liquid Scintillation counter.

(b) The second assay used involved the same reaction mixture and a different detection technique. The reaction mixture was preincubated without enzyme at 37° and at zero time, the dialyzed cell-free extract preparation was added. After 30 minutes at 37°, 25 ul of cold 60% PCA was added and the mixture was placed on ice. The precipitated protein was removed by centrifugation and 40 mg of acid washed charcoal was added to the acid supernatant. The charcoal was spun down and the supernatant was checked for residual radioactivity by adding an aliquot to 2.5 ml Bray's solution and counting in a Nuclear Chicago Mark I.

The charcoal was then washed twice with 50% EtOH containing 1% NH_4OH. The washings were concentrated with an air stream, spotted on 3MM paper and run overnight in solvent A (see Chromatography). The spot containing dCDP and dUMP was eluted in a volume of 1.0 ml and 25 ul of 0.1M Tris Acetate pH 8.0 and 2-4 ul of concentrated NH_4OH were added to bring the pH to approximately 8.0. 1 ul of bacterial alkaline phosphatase was then added to this solution and allowed to incubate for 30 minutes at 37°C. The reaction mixture was then concentrated with an air stream, spotted on 3MM paper and run in solvent A. The UdR and CdR spots were located using uv light and counted on the Actigraph strip counter.

(c) The third assay technique used to measure dCMP deaminase activity employed Silica Gel thin layer chromatography. The assay mixture, contained in a reduced volume of 50 ul, was incubated for 30 minutes at 37°C, stopped by heating at 100°C for 1 minute, treated with 1 ul BAP for 30 minutes at 37°C and then 20 ul was cospotted with authentic cold UdR on Silica Gel. The thin layer sheet was developed in solvent C and the UdR spot was cut out and counted in 2.5 ml Bray's solution.

(d) The fourth assay technique removed the necessity for the BAP treatment. The assay mixture was incubated for 30 minutes at 37°C and stopped in boiling water as in (c) and a 20 ul sample was cospotted with authentic dUMP on PEI TLC and run in 0.1M Formate pH 3.4. The PEI sheet

had been predeveloped in distilled water (see Chromatography). The dUMP spot was ovserved under uv light, cut out, and counted in 2.5 ml Bray's solution.

12. Thymine to Thymidylate Transfer Assay

(a) In order to measure the conversion of thymine to dTMP, the technique of Sherman (1963) was employed.

This assay depended on the formation of negatively charged products which would adsorb to Whatman DEAE (DE81) discs while the unreacted thymine and thymidine would remain unadsorbed and could be washed through the paper disc with distilled water. The paper DEAE discs were prewetted to minimize non-specific binding of thymine or thymidine. The discs were dried and glued to planchets and counted on a Nuclear Chicago gas flow planchet counter.

(b) The thymine -to- thymidylate transfer assay was also carried out using Silica Gel and solvent C measuring both thymidine and thymidylate production. The assay mixture contained the following in 50 ul: Tris Acetate pH 7.6, 20 mM; $MgCl_2$, 2 mM; dCMP, 2 mM; dUMP, 2 mM; dCTP, 1 mM; Thymine-2-^{14}C, 2 mM, and 0.1 mg protein.

After 30 minutes incubation at 37°C, the reaction mixture was stopped by boiling for 1 minute and the precipitated protein was sedimented in the centrifuge. A 20 ul sample was cospotted with authentic cold thymidine and thymidylate on Silica Gel and developed in solvent C. The thymidine and thymidylate spots were observed under uv light, cut out and counted in Bray's solution.

13. Phosphomonoesterase Assay

The reaction mixture contained in 50 ul: Tris Acetate pH 7.5, 20 mM; substrate, 2 mM; and approximately 0.1-0.2 mg of protein. The reaction mixture was incubated for 15 minutes at 37°C and stopped with 0.35 ml of cold 4% PCA. The precipitated protein was centrifuged and 300 ul of the supernatant were sampled and analyzed for inorganic phosphate using the Chen procedure (Chen, Toribara and Warner, 1956). Appropriate controls for baseline phosphate in the dialyzed cell free extract and in the substrates were carried out.

14. Deoxyriboaldolase Assay

Two assays were available for the measurement of deoxyriboaldolase activity. The assay employed measured the decrease in deoxyribose-5-P using the diphenylamine procedure of Burton (1956). An alternate procedure, measuring the acetaldehyde produced by deoxyriboaldolase, employed reduced nicotinamide adenine dinucleotide and alcohol dehydrogenase. However, Lomax and Greenberg (1968) stated that this assay was frequently unfeasible due to the relatively rapid oxidation of NADH in some extracts, especially when the aldolase level was low. These workers recommended the diphenylamine procedure of Burton (1956) for the detection of even very low levels of aldolase activity without background interference.

The reaction mixture contained in 40 ul: Tris Acetate pH 7.5, 50 mM; dR-5-P, 2.5 mM and 0.1-0.2 mg

of protein. The mixture was incubated for 30 minutes at 37°C and stopped with 0.35 ml of cold 4% PCA. The precipitated protein was removed by centrifugation and a sample of the acid supernatant was assayed for loss of deoxyribose using the diphenylamine procedure of Burton (1956).

15. Deoxyribomutase Assay

The basis for this assay was the acid instability of the substrate, dR-1-P, and the acid stability of the product dR-5-P. Thus activity was measured by the loss of inorganic phosphate using the Chen procedure (Chen et al, 1956).

The assay mixture contained in 25 ul: Tris Acetate pH 7.5, 20 mM; dR-1-P, 4 mM; and protein 0.05-0.1 mg. The reaction mixture was incubated for 30 minutes at 37°C and stopped with 200 ul of cold 4% PCA. The precipitated protein was removed by centrifugation and samples of the acid supernatant were removed and analyzed for loss of inorganic phosphate using the Chen procedure (Chen et al, 1956).

RESULTS

1. Initial Studies of Phosphomonoesterase Activity

The phosphomonoesterase assay system has been described previously (see Materials and Methods). Five strains of Escherichia coli were studied initially (B, B-1_{20}, B-1-1_2, K12SH, 15TAU$^-$) using five substrates (dCMP, dUMP, dTMP, dAMP, dGMP). The results of these studies are shown in Table (I).

The greatest phosphomonoesterase activity was found for dUMP and dTMP. The activity for these two substrates was much higher than that for dCMP and 5 to 10 times higher than the activity for dAMP and dGMP. This observation led to the possible inference that although the activities were virtually the same in the mutants and the wild type, the higher activity of dUMP phosphomonoesterase could, under suitable substrate conditions, make dUMP a prime deoxyribosyl donor. The reaction product, UdR, could further react with T in a transfer catalyzed by thymidine phosphorylase (Zimmerman, 1962), to yield TdR. Since thymidylate synthetase which uses dUMP as its substrate is not functional in thymineless mutants, there is a potential dUMP pool for these mutants which does not exist in the wild type.

2. Initial Studies of Transferase Activity

Previous work in this laboratory on the proposed direct transfer of thymine to a donor nucleotide to

TABLE I

Initial Results of Studies of Phosphomonoesterase Activity in Cell Free Extracts of Wild Type and thy$^-$ strains of E. coli

Substrate	Organism				
	B	B-1_{20}	B-1-1_2	K12SH	15TAU$^-_2$
	mumoles/hr/mg protein				
dCMP	<20	60	<20	< 20	< 20
dUMP	450	460	360	110	340
dTMP	460	480	590	160	540
dGMP	70	110	110	<40	110
dAMP	70	50	50	50	70

The complete system (50 ul) contained Tris Acetate pH 7.5, 30 mM; substrate, 2 mM; and approximately 0.1-0.2 mg protein. The reaction mixture was incubated 15 minutes at 37°C and stopped with 0.35 ml of cold 4% PCA. The acid supernatant was analyzed for increase in inorganic phosphate using the Chen procedure (Chen, Torbara, and Warner, 1956).

form thymidylate indicated that dCMP was the most effective donor and that this activity could be stimulated by various mononucleotides (dUMP) and mononucleoside triphosphates (dATP, dGTP, dCTP). This activity also differed between cell-free extracts of mutant cells and wild type cells (Swanson, 1968).

Initial studies of this problem with the DEAE filter disc method indicated that the greatest activity could be observed with a mixture of dCMP and dUMP. These results were inconclusive due to contamination of the substrates, dCMP and dUMP, with their respective nucleosides.

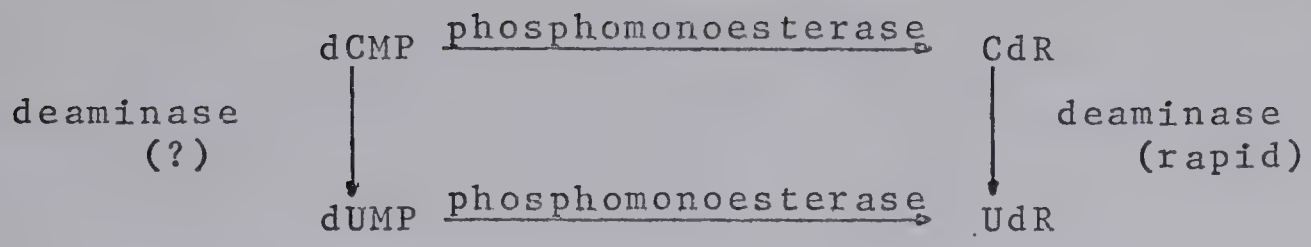
3. Studies of Deoxycytidylate Deaminase Activity

The possibility of the existence of dCMP deaminase had been indicated in this laboratory prior to any investigations for this thesis (Swanson, 1968). The initial assay procedure employed was a modification of that used by Maley and Maley (1960). The procedure involved the acidification of samples taken from the reaction mixture at various times and the separation of the adsorbable substrate, dCMP, from the nonadsorbable product, dUMP, on a Dowex-50 column at pH 2.0. However dCDP and dUMP are electronically equal at pH 2.0 and both dCDP and dCTP would pass through the Dowex-50 without being adsorbed. Also the dephosphorylated product, UdR, would pass through the column. Since the enzyme source was a crude dialyzed cell-free extract, there was considerable conversion of the radioactive substrate, dCMP-2-^{14}C, to the diphosphate

and triphosphate forms. It was found later that the dCMP was also being dephosphorylated and then rapidly deaminated to produce radioactive UdR which was not adsorbed to the column. These alternate routes of production of compounds which would behave like dUMP in this system led to the use of chromatography to separate the products.

In the second assay system (see Materials and Methods), paper chromatography in solvent A was used to separate the radioactivity into three spots (dCMP; dCDP and dUMP; and dCTP). There was also a small quantity of the deoxynucleoside tetraphosphate present. The dCDP and dUMP spot was eluted, treated with BAP, and the treated mixture rechromatographed in the same system to produce two or three spots, UdR, CdR and some partially reacted dCMP and unreacted dUMP. However this procedure was awkward and time consuming as each chromatogram had to be run overnight.

A third procedure (C) was then adopted which combined the BAP treatment and the original reaction mixture without the intermediary step of separation of the dCDP and dUMP spot. The products of the BAP treatment were separated on thin layer Silica Gel with solvent C. The UdR separated cleanly from all other nucleoside and nucleotide components of the system and the technique proved to be satisfactory. However if the reaction was not treated with BAP considerable counts in the UdR area could still be found. The dCMP might be converted to UdR in the two ways shown in the following pathway:



If dCTP was left out of the reaction mixture, more UdR was produced without the BAP treatment than with this treatment. This observation led to the possibility that dCTP was inhibiting CdR deaminase and interfering with the bacterial phosphatase. A typical experiment with accompanying controls is shown in Table (II).

Using polyethylenimine cellulose thin layers and 0.1M Formate pH 3.4, a satisfactory separation of dCDP and dUMP could be achieved. Examination of the reaction with this system and with the Silica Gel system gave results as shown in Table (III).

These results showed that in a reaction mixture without dCTP, the substrate, dCMP, was converted almost quantitatively to the dephosphorylated and deaminated product UdR, rather than to dUMP, the product of dCMP deaminase activity. The addition of dCTP to the system had two effects. One effect was to reduce the CdR deaminase activity and the other was to provide a source of high energy phosphate which could be used by a suitable kinase to phosphorylate UdR to produce dUMP. However the 100 dpm of dUMP activity could also have been produced by dCMP deaminase activity stimulated by the presence of dCTP. By adding a pool of cold dUMP to the reaction mixture, it would be possible to trap

TABLE II

dCMP Deaminase Activity in Escherichia coli B Using Silica Gel Technique

Assay Mixture	B.A.P.	Corrected dpm/20ul/30 min.	Specific Activity mumoles/hr/mg protein
Complete	+	4050	22.6
	-	3100	17.3
-Mg^{++}	+	3300	18.4
	-	600	3.4
-DTT	+	3800	21.2
	-	3600	20.1
-dCTP	+	8800	49.1
	-	10800	60.3
-dCTP, -Mg^{++}	+	7300	40.8
	-	6800	38.0
-dCTP, -Mg^{++}, -DTT	+	13800	77.1
	-	5400	30.1

The complete assay mixture contained in a final volume of 50 ul: Tris Acetate pH 7.6, 20 mM; Mg^{++}, 2 mM; DTT, 1 mM; dCTP, 1 mM; dCMP-2-^{14}C, 1 mM, 0.2 uc; and 0.1-0.2 mg protein. The reaction mixture was treated as described in Materials and Methods. The dpm/20ul/30 min. values are corrected for background activity found in a reaction mixture to which enzyme was not added.

TABLE III

Distribution of Label in Products of dCMP Deaminase Reaction Mixtures With Strains of Escherichia coli

Organism	Assay Mixture	dpm dCMP	dUMP	UdR	dCDP + dCTP
B	complete	22600	100	144000	5000
	$-Mg^{++}$	99000	80	46000	2400
	-dCTP	2500	-	156000	-
	$-Mg^{++}$, -dCTP	6000	-	150000	-
	+dUMP	131000	240	4200	7800
$B\text{-}1_{20}$	complete	90000	290	23000	16000
	$-Mg^{++}$	111000	275	13000	5000
	-dCTP	66000	-	67000	300
	$-Mg^{++}$, -dCTP	93000	-	42000	70
	+dUMP	80000	650	13000	22000
$B\text{-}1\text{-}1_2$	complete	95000	270	16300	20000
	$-Mg^{++}$	126000	160	8300	4100
	-dCTP	117000	-	24000	1100
	$-Mg^{++}$, -dCTP	92000	160	31000	100
	+dUMP	114000	80	7000	11600

dCMP, dCDP and dCTP, and dUMP were measured using the PEI thin layer system in 0.1M Formate, pH 3.4. UdR was measured with the Silica Gel thin layer system and solvent C. The concentration of dUMP was 2 mM.

any dUMP which was formed during incubation. This procedure was carried out and the radioactivity doubled, going from 100 dpm to 200 dpm which was negligible compared to the total radioactivity (176,000 dpm) in the sample. dUMP also appeared to slow conversion of dCMP to UdR, probably by competition at the phosphomonoesterase level.

4. Later Studies of Phosphomonoesterase Activity

From the studies of dCMP deaminase activity, it appeared that there was considerable dCMP phosphomonoesterase activity in fresh dialyzed cell free extracts. Re-examination of phosphomonoesterase activity using fresh dialyzed cell free extracts gave the results shown in table (IV).

Comparison between the members of the B group reveal a 2 fold difference between the mutants and the wild type for dUMP. However this decrease in activity can not be seen in $15TAU_2$ or 5275_2. Activity for other substrates does not vary appreciably. The $B\text{-}1\text{-}1_2$ mutant generally had the lowest activities for all substrates, however this was not reflected by the two other low thymine requirers, 5275 and $15TAU^-$. Thus there appeared to be no major differences in activity for the substrates, dCMP and dUMP, between the thymineless mutants and the wild type.

5. Thymine to Thymidylate Transfer

Initial studies of this proposed reaction were confused due to the presence of contaminants in the substrate solutions. Using cleaned preparations of

TABLE IV

Phosphomonoesterase Activity in Strains of E. coli

Organism	Media Supplement T	Media Supplement TdR	Substrate Activity umuoles/hr/mg protein dUMP	dTMP	dCMP	dAMP	dGMP
K12SH	+		890	1450	450	560	600
K12SH		+	700	1050	220	520	400
$15TAU^{-}_{2}$		+	780	1330	680	820	615
5275_{2}	+		750	1070	640	640	575
5275_{2}		+	410	520	370	300	170
B			270	460	200	210	80
$B\text{-}1_{20}$	+		130	420	180	160	80
$B\text{-}1\text{-}1_{2}$	+		130	260	180	90	50

The composition and treatment of the reaction mixtures has been described previously (see Materials and Methods).

dCMP and dUMP (see Materials and Methods) and an assay involving thin layer Silica Gel and solvent C, the production of both dTMP and TdR could be followed. In all cases except one, no TMP could be detected using dialyzed cell-free extracts of E. coli B. However TdR was produced in all reaction mixtures (Table V).

The reaction mixture which did not contain dCMP produced the most counts in both the dTMP and the TdR areas. This result is in opposition to the reports of Swanson (1968). dTMP was produced only when dCTP was present, possibly because a triphosphate donor was necessary to produce thymidylate as a result of thymidine kinase activity. dUMP appeared to be the best substrate in initial experiments. Since considerable dUMP phosphatase activity had been found in previous work, it was feasible that the activity was the result of UdR conversion to TdR. This seemed logical because 2 to 3 times more TdR than dTMP had been produced in a reaction mixture containing dUMP as the substrate. To test this hypothesis, similar experiments were carried out using the nucleoside rather than the nucleotide. The results are shown in Table (VI).

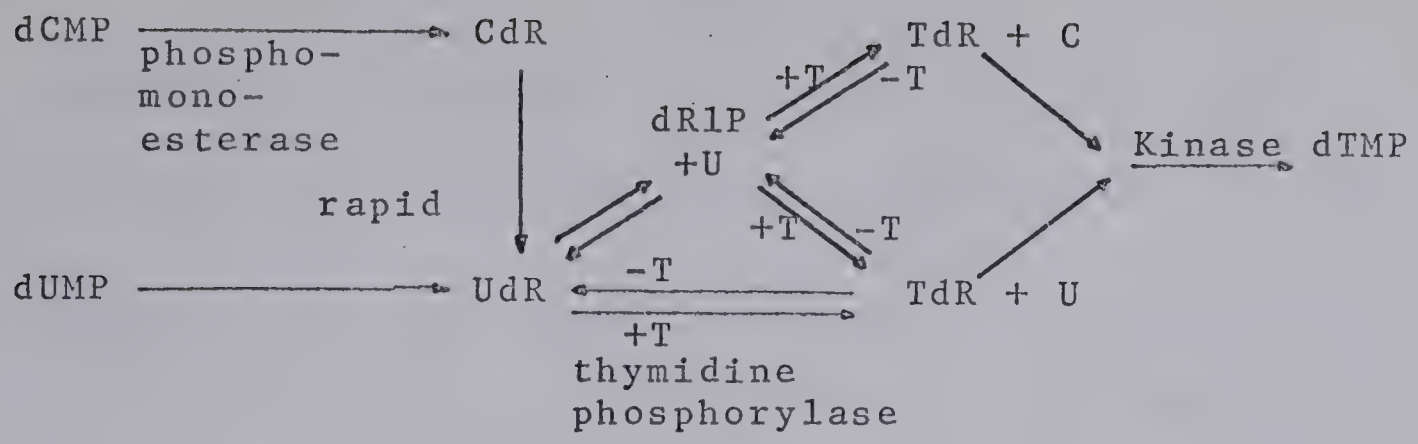
From these results it appeared that the enzymes responsible for the production of thymidylate were dUMP phosphomonoesterase, thymidine phosphorylase and thymidine kinase. The probable reaction sequence is shown below.

TABLE V

Thymine to Thymidylate Conversion in Dialyzed Cell Free Extracts of E. coli B

Assay	Corrected DPM/20ul/30 min. TdR	dTMP
Complete	3150	<50
$-Mg^{++}$	2300	"
-dCMP	5000	1900
-dUMP	2300	<50
-dCTP	4400	"
$-Mg^{++}$, -dCTP	4500	"
-dCMP, -dCTP	4300	"
-dUMP, -dCTP	1300	"

The complete reaction mixture contained the following in 50 ul: Tris Acetate pH 7.6, 20 mM; $MgCl_2$, 2 mM; dUMP, 2 mM; dCMP, 2 mM; dCTP, 1 mM; T-2-^{14}C, 2 mM, 2 uc/um; protein, 0.15 mg. The mixture was incubated for 30 minutes at 37°C and stopped by boiling for one minute. The precipitated protein was removed by centrifugation and a 20 ul sample of the supernatant was removed and cospotted with cold TdR and dTMP on Silica Gel. After the thin layer sheet was developed in solvent A, the spots were isolated, cut out, and counted in 2.5 ml Bray's.



In the absence of triphosphates, UdR and dR-1-P appeared to be the best deoxyribose donors. In the presence of dCTP, CdR, UdR, and dR-1-P all appeared to be good donors. In the presence of ATP, UdR and CdR serve equally well as deoxyribose donors. Thus thymidine formation can take place in Escherichia coli B cell free extracts by the salvage pathway rather than the de novo pathway using thymidylate synthetase. This finding agreed with the work of Mantsavinos and Zavenhof (1961).

The production of thymidylate was greatest in reaction mixtures which had dR-1-P as the substrate and a triphosphate (ATP or dCTP) present. However considerable production was also found in the dCMP + ATP reaction mixture with E. coli B-1-1 cell free extract. The multiple pathways involved in dTMP production prevented further investigation of this low activity.

6. Deoxyriboaldolase Activity

A comparison of several strains of Escherichia coli was made to determine the amount of deoxyriboaldolase activity present. It was found that both of the wild types (K12SH, B) and both of the low requiring

TABLE VI

Comparison of Substrates For Thymine to Thymidylate Conversion by Cell Free Extracts of Strains of E. coli

	Corrected DPM/20ul/30 min.					
	E. coli B		B-1$_{20}$		B-1-1$_2$	
Substrate	TdR	dTMP	TdR	dTMP	TdR	dTMP
dUMP	1000	-	1600	110	1800	100
UdR	60000	470	21000	140	16500	-
dCMP	2300	-	1400	50	2250	-
CdR	16000	30	23000	250	4000	-
dR1P	68500	500	24000	100	27000	450
dUMP + ATP	3400	140	4700	200	3000	1000
UdR + ATP	70000	310	43000	400	35000	400
dCMP + ATP	1600	220	100	1250	6500	2000
CdR + ATP	71000	130	30000	-	2200	-
dR1P + ATP	46500	1000	34000	450	20000	1100
dUMP + dCTP	2600	200	2400	800	1200	1250
UdR + dCTP	32000	100	60000	900	50000	1000
dCMP + dCTP	1100	330	300	750	3400	500
CdR + dCTP	71000	300	29000	200	71000	200
dR1P + dCTP	68500	1500	30000	900	30000	1140

The assay mixture contained the following in 50 ul: Tris Acetate pH 7.6, 20 mM; $MgCl_2$, 2 mM; T-2-^{14}C, 2 mM, 2 uc/umole; substrate, 2 mM; ATP or dCTP, 1 mM; and 0.10 mg protein.

strains (15TAU$^-$, 5275) contained deoxyriboaldolase activity comparable to that found by Breitman and Bradford (1967). These results are shown in Table (VII). It was of special interest that both 15TAU$^-$ and 5275 possessed the aldolase activity and yet both were low thymine requirers. Other workers (Hoffee, 1968; Breitman and Bradford, 1967) have reported low thymine requiring strains which do not possess deoxyriboaldolase activity.

7. Deoxyribomutase Activity

Because both low thymine requirers used in these experiments possessed normal basal levels of deoxyriboaldolase activity, the deoxyribomutase levels of these strains came under question. It appeared reasonable that the low thymine requirement resulted from increased dR-1-P levels due to the inability of the low thymine requirers to catabolize deoxyribose. Since the aldolase activity was normal for these double mutants, it was possible that the mutase activity was missing. The results of the deoxyribomutase assay are shown in Table (VIII), and are in agreement with results published by Breitman and Bradford (1968).

E. coli K12SH did not show induction of deoxyribomutase activity after growth in a medium containing thymidine whereas strain B did, increasing from 235 to 560 mumoles/hr/mg of dR-1-P converted to dR-5-P. However the level of deoxyribomutase activity in K12SH in the thymine supplemented medium appears to be the induced

TABLE VII

Deoxyriboaldolase Activities of 4 Escherichia coli Strains

Organism	OD_{600}	mumoles/hr/mg protein
K12SH	0.68	2,560
B	0.35	2,740
$15TAU^-$	1.1	1,000
5275_2^-	0.62	2,400
Control - Enzyme	1.4	-

The reaction mixture contained the following in 40 ul: Tris Acetate pH 7.5, 50 mM; dR-5-P, 2.5 mM; and approximately 0.1 to 0.2 mg protein. The reaction mixture was stopped after 30 minutes at 37°C with 0.35 ml of cold 4% PCA. The protein-free acid supernatant was analyzed for loss of deoxyribose-5-P using the diphenylamine procedure of Burton (1956).

TABLE VIII

Deoxyribomutase Levels in Cell Free Extracts of Strains of E. coli After Growth On Either Thymine or Thymidine Supplemented Media

Organism	Supplement T	Supplement TdR	Corrected OD_{820}	mumoles/hr/mg protein
K12SH	+		0.52	610
		+	0.52	560
B	+		0.55	235
		+	0.50	560
15TAU		+	0.56	<85
5275	+		0.57	<45
		+	0.56	<45
Control			0.565	

The measurement of the conversion of the substrate dR-1-P to dR-5-P was based on the acid instability of dR-1-P, while the product dR-5-P as well as glyceraldehyde-3-P are acid stable. Thus the conversion is followed by measuring the loss of inorganic phosphate in the reaction mixture

The thymidine concentration in the medium was 1 mM.

level. Both low requiring strains showed low levels of deoxyribomutase activity and strain 5275 did not show an induction of activity similar to that of E. coli B when grown in a medium containing thymidine.

DISCUSSION

There were no significant differences in phosphomonoesterase activity found in any of the wild type, high- or low-requiring strains. The activities for the various strains for a given substrate were all within a range which appeared to be much higher than necessary for supplying deoxyribose for production of dTMP during normal DNA synthesis. The phosphomonoesterase activities ranged for dUMP from 400 to 900 mumoles/hr/mg protein. The B family of mutants displayed a lower set of activities for all five substrates (dCMP, dUMP, dTMP, dAMP, dGMP). Normal DNA production can be supported in the wild type by thymidylate synthetase with a specific activity of 15 mumoles/hr/mg protein (Harrison, 1965). Thus it appeared that phosphomonoesterase activity was not a limiting step in the supply of deoxyribosyl donors. The inactivity of thymidylate synthetase in high and low thymine requirers should result in an increase in the amount of dUMP available within the cell. The dephosphorylation of dUMP results in the production of UdR and this nucleoside can serve as a substrate for thymidine phosphorylase (Razzell and Casshyap, 1964).

Prior to the establishment of an equilibrium after the loss of thymidylate synthetase, dTTP levels would fall and result in an accumulation of dATP, dGTP and dCTP. Degradation of these deoxynucleoside triphosphates would

increase the deoxyribose pool and hence the synthesis of thymidine if an external source of thymine were present.

Therefore higher levels of these deoxynucleoside triphosphates could be expected in thymine requiring mutants. Neuhard (1966) found that when thymine requiring mutants were starved for thymine the dATP and dCTP pool size increased. With the establishment of equilibrium both the deoxynucleoside triphosphates and the dUMP pool could be expected to contribute the required deoxyribose for dTMP production.

The results displayed in Table VI show that UdR and dR-1-P are equally good substrates for thymidine phosphorylase. Razzell and Casshyap (1964) reported that CdR was not a substrate of pure thymidine phosphorylase. In the crude cell free extract used in these experiments CdR must have been converted first to UdR by the active CdR deaminase present. Thus in crude cell free extracts it was possible for CdR to support the synthesis of TdR.

From the results for *E. coli* B shown in Table VI it appeared that the addition of a nucleotide (dUMP, dCMP) as a substrate required phosphomonoesterase activity to convert it to a nucleoside prior to use. Only when a nucleoside or deoxynucleoside triphosphate was present, was there any conversion of thymine to thymidylate. Reaction mixtures containing dR-1-P and ATP or dR-1-P and dCTP produced the largest amount of thymidylate. In *E. coli* B-1_{20} and B-1-1_2 cell free extracts there appeared

to be some thymidylate formation in assay mixtures containing dCMP and ATP or dCMP and dCTP.

Deoxycytidylate deaminase could not be unequivocally demonstrated in any cell free extract at any time. The sensitivity of the assay employed was about 10^{-2} mumoles of dUMP. This limit was due to the low specific activity of the radioactive substrate dCMP-2-^{14}C (2 uc/umole). Higher specific activities would result in increased sensitivity. The fourth dCMP deaminase system used had a higher sensitivity than any other system described previously (Keck et al, 1960; Maley and Maley, 1966) and was well suited for the examination of crude cell free extract enzyme sources.

Thus no major differences were found in any of the enzymatic activities examined which could lead to an increased pool of dR-1-P by production of this compound.

The nature of the second mutation which controls the quantitative thymine requirement provided a more interesting problem. The mutation (tlr or thy-R) has been mapped in E. coli K12 (Okada, 1966; Alikhanian et al, 1966) and in S. typhimurium (Eisenstard et al. 1968) and is located some distance from the thy locus as described previously (see Introduction). Several reports have indicated that the mutation is involved with deoxyribose metabolism, particularly with the catabolic enzyme, 5-phosphodeoxyriboaldolase (Hoffee, 1968; Breitman and Bradford, 1967). This locus (tlr) is also involved in the

control of another phenotype, deoxyriboside sensitivity (Hoffee, 1968; Lomax and Greenberg, 1968).

The investigation of aldolase levels in $15TAU^-_2$ and 5275_2 for this thesis revealed normal basal levels for this enzyme. However it was found that both of these mutants possessed low levels of phosphodeoxyribomutase (Table VIII). This finding was in agreement with the work of Breitman and Bradford (1968) who reported that two low thymine requiring strains ($15T^-$ and strain I) both possessed similarly low levels of mutase activity. The deficiency of strain I was maintained after the back-mutation to thymine independence. Both strains were not inducible for deoxyriboaldolase or thymidine phosphorylase which agrees with the work of Hoffee (1968) who used S. typhimurium. Breitman and Bradford (1968) also report that their findings on the mutase deficiency have been confirmed by personal communication with A. Munch-Peterson (K12) and R.C. Bockrath ($15T^-$ derived mutants).

The mutase deficiency of E. coli 5275 helped to explain the finding of Razzell and Casshyap (1964) that this organism was not inducible for thymidine phosphorylase. Externally applied dR-1-P could not be converted intracellularly to dR-5-P, the inducer of both deoxyriboaldolase and thymidine phosphorylase. That dR-5-P is the inducer of these enzymes was shown by Barth et al (1968) using a mutase deficient strain (E. coli K12:

P152) which was not inducible for either of these enzymes. Their aldolase deficient strain (CR34) was constitutive for thymidine phosphorylase and the mutase because it possessed an increased pool of dR-5-P, being unable to catabolize this compound. The aldolase and mutase deficient strains both excreted free deoxyribose into the medium during normal growth (Barth et al, 1968). When either of these deficiencies were coupled with the loss of thymidylate synthetase, the double mutant gained an enhanced ability to synthesize thymidine from thymine due to the enlarged deoxyribose pool. When a double mutant (E. coli 70V3-462) was starved for thymine, excretion of deoxyribose into the medium also occurred (Breitman and Bradford, 1964). Thus it appeared that loss of the ability to catabolize deoxyribose through either an aldolase or a mutase deficiency resulted in an increased deoxyribose pool and a lowered thymine requirement.

Ahmad et al (1968) have postulated an operon in E. coli which contains thymidine phosphorylase, purine nucleoside phosphorylase, deoxyriboaldolase and perhaps deoxyribomutase, located to the left of the thr locus. These enzymes have in common the inducer, dR-5-P.

Lomax and Greenberg (1968) have also reported a clustering of genes concerned with thymidine catabolism on the left side of the thr locus. These workers proposed that the deo A gene controlled thymidine phosphorylase, the deo C gene controlled the aldolase and

that the deo B gene controlled the mutase. The mutase-deo B relationship had not been verified by enzyme assay. The close linkage of these genes (deo A, B, and C) and the biochemical relationship of their products indicated that these genes may constitute an operon as proposed by Ahmad et al (1968).

In conclusion it appears that the high thymine requirement is the result of an internal concentration of dR-1-P which is too low to maintain a rate of synthesis of thymidylate to permit DNA synthesis to occur at its normal rate when the external thymine concentration is low. The low thymine requirement appears to result from blockage of further deoxyribose catabolism at either the mutase or the aldolase level which permits the deoxyribose pool to increase thus enhancing the rate of synthesis of thymidine by thymidine phosphorylase. Revertants from the tlr strains of E. coli such as $15T^-$ retain the ability to incorporate exogenous thymine (Crawford, 1968). However revertants from high-thymine-requiring strains will not incorporate exogenous thymine unless a deoxyribose donor (AdR) is supplied in the medium (Budman and Pardee, 1967). Thus it appears that the availability of deoxyribose donors is the key to the problem of quantitative thymine requirements.

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